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The effect of diet on protein concentration, hypopharyngeal gland development and virus load in worker honey bees (*Apis mellifera* L.)

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ABSTRACT

Elucidating the mechanisms by which honey bees process pollen vs. protein supplements are important in the generation of artificial diets needed to sustain managed honeybees. We measured the effects of diet on protein concentration, hypopharyngeal gland development and virus titers in worker honey bees fed either pollen, a protein supplement (MegaBee), or a protein-free diet of sugar syrup. Workers consumed more pollen than protein supplement, but protein amounts and size of hypopharyngeal gland acini did not differ between the two feeding treatments. Bees fed sugar syrup alone had lower protein concentrations and smaller hypopharyngeal glands compared with the other feeding treatments especially as the bees aged. Deformed wing virus was detected in workers at the start of a trial. The virus concentrations increased as bees aged and were highest in those fed sugar syrup and lowest in bees fed pollen. Overall results suggest a connection between diet, protein levels and immune response and indicate that colony losses might be reduced by alleviating protein stress through supplemental feeding.

1. Introduction

Honey bee colony losses are greater now than any time in recent history (vanEngelsdorp et al., 2007, 2009). The causes for colony deaths have been attributed to parasitic mites (Amdam et al., 2004) and disease, but reasons for other losses such as those dying from colony collapse disorder (CCD) have been elusive (vanEngelsdorp et al., 2009). Perhaps an underlying factor contributing to colony losses is inadequate nutrition. A steady supply of pollen insures the growth of colonies because it provides protein to adult bees and stimulates brood rearing. Colonies with low nutritional reserves have reduced brood rearing (Keller et al., 2005; Mattila and Otis, 2007; DeGrandi-Hoffman et al., 2008) and workers that transition from nest activities to foraging earlier in their adult life (Schultz et al., 1998; Toth et al., 2005; Toth and Robinson, 2005). Worker longevity is affected by the age of first foraging (Guzman-Novoa et al., 1994; Rueppell et al., 2007). Workers that become foragers earlier in life die sooner than their nestmates that perform tasks in the hive. Thus, colonies with limited protein intake decline from the combination of reduced brood rearing and a shorter lifespan for adult workers. If parasitic mites and pathogens are present, the population decline can be even more severe so that the colony perishes.

Honey bees rely on pollen as their source of protein, lipids, sterols, vitamins, minerals and certain carbohydrates (Todd and Betherick, 1942). Nectar is primarily a carbohydrate source, but can contain some amino acids and lipids (Percival, 1961; Baker and Baker, 1975). Some digestion of pollen occurs in the midgut, but the primary means by which the nutrients from pollen are made available to the colony is its conversion to worker jelly. The conversion occurs in the paired food glands called hypopharyngeal glands (HPG) located in the frontal area of the worker's head (Hrassnigg and Crailsheim, 1998). The glands are comprised of acini that produce the protein-rich worker jelly that is fed to larvae of all castes and to the queen. Nurse bees distribute the jelly to nestmates through trophallactic interactions (Crailsheim, 1991). Via this system, the nutrients from the pollen are circulated throughout the colony.

In addition to the need for pollen in brood rearing and to optimize worker longevity, nutrition (particularly protein availability) is a key factor in resistance to pathogens (Ford et al., 2001; Kaminogawa and Nanno, 2004; Ritz and Gardner, 2006; Rowley and Powell, 2007). Encapsulation, phenyloxidase, and lysozyme activity are enzyme-based immune responses to foreign invaders such as virus and to wounding and are affected by protein deficiencies (Siva-Jothy et al., 2005; Lee et al., 2006). In honey bee colonies, protein deficiencies that affect the immune response could accelerate the spread of disease among nestmates and cause pathogen levels to increase so that adult longevity and survival are reduced. Thus, what began as a nutritional deficiency could develop into colony loss from disease.

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Pollen is not always available to colonies, so in managed hives, protein supplements are fed to bees to stimulate brood rearing and prevent colony populations from declining (e.g. Nabors, 2000; Mattila and Otis, 2006a). The supplements might not contain pollen, but instead have protein derived from sources such as soy or whey. Ideally, feeding protein supplements causes a flow of nutrients through a colony that is similar to pollen because the supplement stimulates the HPG of young bees to produce worker jelly. In some instances, feeding colonies protein supplements can cause colony populations to grow at rates that are similar to those fed pollen (DeGrandi-Hoffman et al., 2008; Mattila and Otis, 2006a).

Whether protein supplements are metabolized in a similar manner to pollen is not known. Here we compare protein levels and HPG acini size of young worker bees fed either bee-collected pollen or a protein supplement. The effect of nutrition on immune response was indirectly inferred by comparing virus concentrations over time in workers fed different diets. Specifically, we examined the effects of diet on titers of deformed wing virus (DWV). This positive stranded RNA virus causes morphological deformities (i.e., stick wings) and early death in newly emerged adult bees (Bowen-Walker and Gunn, 1998). DWV is one of the most widespread and prevalent viral infections of *A. mellifera* worldwide (Allen and Ball, 1996; Ellis and Munn, 2005). There is a significant correlation between DWV titers and overwintering colony losses (Highfield et al., 2009), and along with other picorna-like viruses it is associated with colony losses from CCD (Johnson et al., 2009).

We compared the effects of a protein supplement with pollen and a protein-free feeding regime on newly emerged worker bees placed in cages. There are strengths and limitations with using caged bees rather than colonies in studies such as ours. A limitation is that it does not account for colony-level interactions that might affect worker metabolism and immune response. However, a strength of this method is that the cages eliminate variability from differences that occur among colonies in terms of incoming nectar and pollen, size of brood areas, queen egg laying rates, and colony population size and age structure. We interpret the results with respect to the limitations of cages, but also discuss how our results might be extended to colony-level effects of supplemental protein feeding on colony health and population growth.

2. Materials and methods

The study was conducted at the Carl Hayden Bee Research Center, Tucson Arizona, U.S.A. from June through July of 2008. All bees used in the study were from European honey bee (*Apis mellifera ligustica*) colonies headed by commercially produced and mated European queens (Kona Queens, Captain Cook, HI). We conducted three trials over a 6-week period. A trial lasted for 11 days. In each trial we established 5 cages per diet treatment for a total of 15 cages per trial. A different source colony for the bees was used in each trial. Protein concentrations and HPG measurements were made using three bees collected randomly from each cage during each sampling interval.

2.1. Preparation of diets

Bees were fed ad lib either pollen patty or a protein supplement that lacks pollen (MegaBee® patty). MegaBee was chosen as a protein supplement because in full sized queen right colonies it is consumed at rates that are similar to pollen patties (DeGrandi-Hoffman et al., 2008). Pollen patties contained a mixture of pollens collected by bees in the Sonoran desert in Arizona, USA. The pollen was collected less than 12 months before the start of the study, and kept frozen until using it for making the patties. After the patties were made, they were kept frozen until fed to the bees. Previous studies examining the degradation of stored pollen over time indicate that the nutritional value does not decline in the first year of storage so that the lifespan of workers in cages (deGroot, 1953) or brood production in colonies is reduced (Hagedorn and Moeller, 1968; Haydak, 1970).

Pollen patties were made by combining pollen with equal parts (by weight) of granulated sucrose, Drivert sugars (a mixture of equal parts of sucrose and dry fructose) and tap water (DeGrandi-Hoffman et al., 2008).

2.2. Feeding diets

At the start of each trial, frames with sealed worker brood were put in a temperature controlled room (32–34 °C). When the adults emerged, they were pooled and transferred into cages. Twenty workers were sampled from the pool of newly emerged bees (five each for estimates of protein and HPG development and 10 for detection and quantification of virus) prior to the treatment to establish a baseline for protein concentration, HPG development and virus titer (day 0 samples) for the trial. Then, an average of 124 ± 2 newly emerged bees were transferred into each of the plexiglas cages (15 11.5 cm × 7.5 cm × 16.5 cm) used per trial. All bees in the cages were <24 h old at the start of the trial. The bees were fed pollen patty, or a protein supplement. Vials with distilled water and high fructose corn syrup also were provided to treatment and control cages. Control cages received no pollen patty or protein supplement. We calculated the amount of diet consumed during the 11-day period of each trial by weighing the pollen and protein supplement patties at the beginning and end of the trial. Bees were sampled from all cages after feeding for 4, 7 and 11 days. Sampled workers were kept frozen until protein and HPG analysis.

2.3. Protein analysis

Protein concentrations were estimated by removing the heads from three worker bees sampled per cage, and homogenizing them individually in 2 ml Eppendorf microcentrifuge tubes (Eppendorf North America, Westbury, NY) containing 75 mM phosphate buffer solution (pH 7.4). The sample was centrifuged and analyzed for protein content using the 500-0202 Quick Start Bradford Protein Assay Kit 2 (Bio-Rad Laboratories, Hercules, CA) (Sagili et al., 2005; Sagili and Pankiw, 2007). The protein concentrations in the pollen cake and the protein supplement were estimated using the same protein assay kit. Standard curves to estimate protein concentration in the samples were prepared using bovine serum albumin (BSA). Protein absorbance was measured at 595 nm using a Biotek Synergy HT spectrophotometer.

2.4. Hypopharyngeal gland measurements

HPG were removed from 3 bees per cage. The glands were placed in a Petri dish with wax depressions containing water. A photo of the glands was taken using a Leica MX12 microscope with a Leica DC-300 camera and Leica IM-50 image manager software program (v 1.2). For calibration, a photograph of a 1 mm line was taken at the same magnification used for observing the HPG. The 2-dimensional areas of five acini were measured for each HPG sample using the Photoshop (Adobe) pixel counting routine. The area of each acini was estimated by the equation: $N_a \times (N_{mm})^{-2}$ where N_a = the number of pixels for each acini and N_{mm} = the number of pixels in a 1 mm line that is 1 pixel wide. N_{mm} was squared to estimate the number of pixels in a 1 mm × 1 mm area and multiplied by 0.001 to convert it to microns. The size of the five acini was averaged and used as acini measurement for the bee.

2.5. Virus detection—RNA extraction

Three bees were collected from each cage at each time point prior to and after the treatments (days 0, 4, 7, and 11). Total RNA was extracted from individual bees. Each bee was homogenized in TRIzol Reagent, a solution of guanidine isothiocyanate and phenol for RNA extraction (Invitrogen, Carlsbad, CA), and total RNA was extracted following the manufacturer's standard protocol. After isopropanol precipitation, the RNA pellet was dissolved in diethylpyrocarbonate (DEPC)-treated water in the presence of Ribonuclease Inhibitor (Invitrogen, Carlsbad, CA) and stored at −80 °C for molecular analyses.

2.6. Virus detection by RT-PCR

RNA samples extracted from bees collected prior to treatments were tested for the presence of seven bee viruses: acute paralysis bee virus (APBV), black queen cell virus (BQCV), chronic paralysis bee virus (CPBV), deformed wing virus (DWV), Israeli Acute Paralysis Virus (IAPV), Kashmir bee virus (KBV), and sacbrood bee virus (SBV) by RT-PCR. Access RT-PCR system (Promega, Madison, WI) was used for RT-PCR reaction according to the manufacturer's instructions. The reaction was performed in a total volume of 25 μl with a final concentration of 1× of AMV/Tfl reaction buffer, 0.2 mM of dNTP, 1.0 μM of sense primer, 1.0 μM of antisense primer, 2 mM of $MgSO_4$, 0.1 unit of AMV reverse transcriptase, 0.1 unit of T*fl* DNA polymerase and 500 ng of total RNA. Amplification was undertaken using the PTC-100 DNA Engine (MJ Research, Waltham, MA) with the following thermal cycling profiles: one cycle at 48 °C for 45 min for reverse transcription; one cycle of 95 °C for 2 min; 40 cycles at 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 2 min; one cycle of 68 °C for 7 min. Negative (H_2O) and positive controls (previously identified positive sample) were included in each run of the RT-PCR reaction. PCR products were electrophoresed in 1% agarose gel containing 0.5 μg/ml ethidium bromide and visualized by UV transillumination. A 100-bp DNA ladder (Invitrogen) was included as a standard on each gel.

2.7. Virus quantification by real-time quantitative RT-PCR

RNA samples that tested positive for virus infection by conventional RT-PCR were subjected to real-time quantitative RT-PCR assay (qRT-PCR). The assay was performed for virus quantification using Stratagene Mx3000P spectrofluorometric thermal cycler operated by MxPro qPCR software. The primer pair specific for DWV was used for virus quantification because DWV was the only virus found in bees prior to diet treatments. The DWV primer pair (Forward: 5′-CGAAACCAACTTCTGAGGAA-3′; Reverse: 5′-GTGTTGATCCCTGAGGCTTA-3′) amplifies a 174-bp fragment. The housekeeping gene, β-actin, was used as a reference gene. The β-actin primer pair (Forward: 5′-AGGAATGGAAGCTTGCGGTA-3′ and Reverse: 5′-AATTTTCATGGTGGATGGTGC-3′) amplifies a 181-bp fragment. Reactions were carried out in a 50-μl reaction volume containing 25 μl of 2× Brilliant® SYBR® Green QRT-PCR Master Mix (Stratagene, La Jolla, CA), 0.4 μM each of forward and reverse primers, and 1 μg of template RNA. The thermal profile for the one step RT-PCR was as follows: one cycle at 50 °C for 30 min, one cycle at 95 °C for 10 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 30 s. After amplification, a melting curve analysis was performed to determine the specificity of the PCR products. The PCR products were incubated for 1 min at 95 °C, ramping down to 55 °C at a rate of 0.2 °C/s. The dissociation curve was constructed using 81 complete cycles of incubation where the temperature was increased by 0.5 °C/cycle, beginning at 55 °C and ending at 95 °C. The expected size of the PCR product was confirmed by 1.5% agarose gel electrophoresis and subsequent visualization with ethidium bromide. Negative controls (no reverse transcriptase and no template) were included in each run of the reaction and yielded no products.

2.8. Interpretation and validation of the qRT-PCR output

Virus levels in a subsample of nine bees taken from the 15 bee sample (three bees each from five cages per diet treatment) were quantified based on the value of the cycle threshold (C_t). This was repeated for bees sampled at each time interval (4, 7, and 11 days). Data were analyzed using the comparative C_t method ($\Delta\Delta C_t$) and results were expressed as the fold difference in expression. The average C_t value (ΔC_t) of each treatment group was normalized using the C_t value corresponding to the endogenous control, β-actin following the formula: ΔC_t = Average C_{tDWV} − Average $C_{t\beta\text{-actin}}$. The ΔC_t value of each group at different time points after diet treatment was compared with a calibrator which represented the minimal virus level to derive $\Delta\Delta C_t$. The fold-change in the virus concentration of each treatment group (trt) at different time points post treatment ($Fc(trt)_t$) was calculated using the formula: $(Fc(trt)_t) = 2^{-\Delta\Delta C_t}$.

In order to qualify the use of comparative C_t method ($\Delta\Delta C_t$) for DWV quantification, the validation experiment was performed to demonstrate that efficiencies of DWV and β-actin amplification in the real-time qRT-PCR were approximately equal Nine five-fold serial dilutions (ranging from 10^3 ng to 2.56×10^{-3} ng) of total RNA extracted from a DWV infected bee were used for DWV and β-actin qRT-PCR and run in triplicate. The standard curves of the DWV and β-actin were constructed by plotting the initial quantities of serial diluted total RNA against the corresponding threshold value (C_t). Relative efficiencies of DWV and β-actin amplification were compared.

2.9. Statistical analysis

Average consumption of pollen and protein supplement per cage during each trial was compared using a two-way analysis of variance with diet type and trial as factors. An average protein concentration per bee or size of acini was estimated for each diet treatment using the measurements from the three bees sampled per cage for each factor. The averages were entered into a general linear model that included diet, age of bee and trial. Since differences among trials were detected, protein content in bees and size of acini sampled at different ages was analyzed within each trial with a repeated measures analysis of variance with age of bees and diet as factors. In all cases, Bonferroni adjustment of confidence intervals was selected as the post hoc procedure for mean comparisons (Field, 2005).

We used linear regression analysis to test for a relationship between the amount of diet consumed and the amount of protein detected in workers fed either pollen or protein supplement. The protein concentration detected in the bees was summed for the three sampling intervals (days 4, 7, and 11) for each cage and was used as the dependent variable. The amount of diet consumed by the bees in the cage was the independent variable. Similarly, we used linear regression analysis to test for a relationship between the amount of diet consumed and the size of acini in bees fed the different diets. The size of acini from the bees sampled from each cage was summed across all age classes and used as the dependent variable. The total amount of food consumed by the bees in the cage was the independent variable (Field, 2005). Comparisons in the proportion of workers where DWV was detected at 4, 7, and 11 days based on the diet they were fed were made using *z*-tests with 95% confidence intervals.

3. Results

In Trial-1, bees being fed the protein supplement escaped from one of the cages between days 4 and 7. Estimates of diet

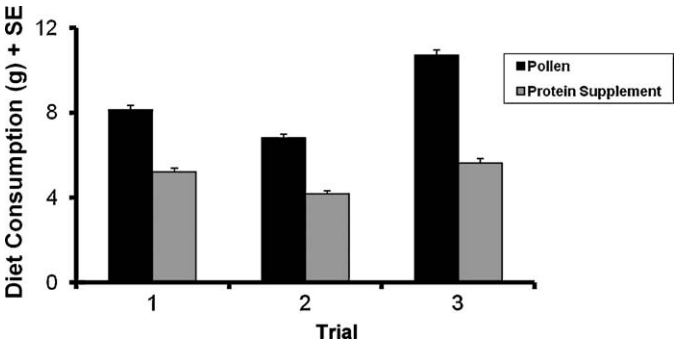

Fig. 1. The average amount (grams) of pollen and protein supplement patty consumed by caged worker honey bees during an 11-day period. In each trial, bees consumed significantly ($p < 0.05$) more pollen than protein supplement.

consumption, acini size, and protein concentration in this trial were made using data collected from the remaining 4 cages.

3.1. Diet consumption

Bees consumed significantly different amounts of pollen and protein supplement both within and between trials (diet type: $F = 481.82$, d.f. = 1, 23, $p < 0.0001$; trial: $F = 23.07$, d.f. = 2, 23, $p < 0.0001$; interaction of diet and trial: $F = 12.9$, d.f. = 2, 23 $p < 0.0001$). In all trials, bees consumed significantly more pollen patty than protein supplement (Fig. 1).

3.2. Protein analysis

The concentration of protein in the pollen patties was similar to that of the protein supplement (pollen patty 289 μg/ml, protein supplement: 225 μg/ml; $t = 1.51$, $p = 0.2$, d.f = 4). Bees that were ≤24 h old had similar concentrations of protein to those that were 4 days old (Fig. 2).

The amount of protein measured in the bees as they aged was affected by diet, age, and trial (diet type: $F = 70.24$, d.f. 2, 116, $p < 0.0001$; age: $F = 4.94$, d.f. = 2, 116, $p = 0.008$; trial: $F = 6.95$, d.f. = 2, 116, $p = 0.001$) (Fig. 2). Interactions between age and diet and age and trial also were significant ($F = 7.95$, d.f. = 4, 116, $p < 0.0001$ and $F = 6.96$, d.f. = 4, 116, $p < 0.0001$ respectively). There were significant effects due to trial, so separate repeated measures ANOVA were conducted using data from each trial.

Pairwise comparisons of means estimated from the repeated measures analysis of variance using Bonferroni adjusted confidence intervals (*post hoc* comparisons of the repeated measures analysis of variance) indicated that in trial-1, the amount of protein was greater in bees fed either pollen or protein supplement compared with controls ($p = 0.012$, $p = 0.01$ respectively). Amounts of protein in 4- and 7-day old bees were significantly higher than in day 11 bees ($p = 0.006$ and $p = 0.03$ respectively). In trials-2 and -3, the results were similar to trial-1 in that protein amounts were highest in bees fed either pollen or protein supplement (trial-2: $p = 0.014$, $p = 0.006$, trial 3: $p = 0.001$, $p = 0.001$ respectively). However, the protein amounts detected in the workers fed each diet did not differ with age ($p > 0.05$). Interaction between diet type and age of worker was not significant in any of the analyses of individual trials.

3.3. Area of acini in hypopharyngeal glands

The HPG of workers that were ≤24 h old had started to develop before we put the bees in the cages and fed them the different diet treatments (Fig. 3). In all trials, the size of the acini decreased in control workers and increased or remained similar in size to the ≤24 h old bees in those fed pollen or protein supplement.

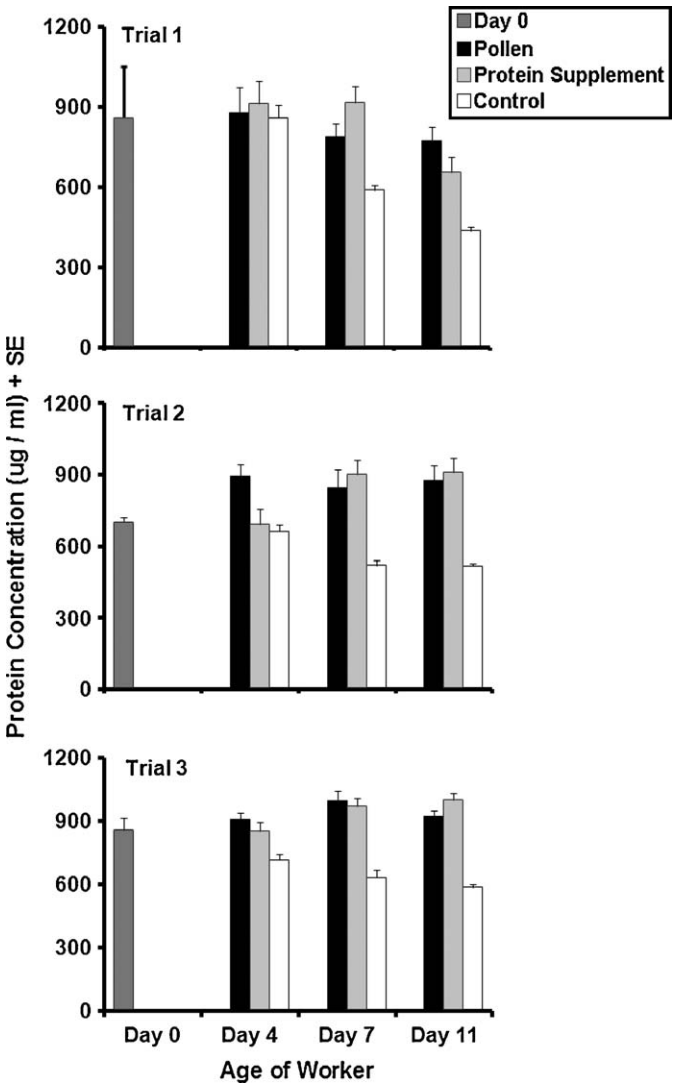

Fig. 2. Average concentration of protein in the head capsules of worker honey bees of different ages fed pollen, protein supplement, or a diet of water and high fructose corn syrup (Control). Each mean was estimated from protein measurements of 15 worker bees except for day-0 where means were estimated using 5 worker bees. Protein concentrations were greater in bees fed either pollen or protein supplement compared with controls ($p = 0.012$, $p = 0.01$; trial-2: $p = 0.014$, $p = 0.006$, trial 3: $p = 0.001$, $p = 0.001$). Amounts of protein in 4- and 7-day old bees were significantly higher than in day-11 bees in trial-1 only ($p = 0.006$ and $p = 0.03$; respectively). In trials-2 and -3, protein amounts detected in the workers fed each diet did not differ with age ($p > 0.05$).

The average area of acini of the HPG of workers fed the different diets depended upon the type of diet, age of the worker and trial (diet: $F = 48.06$, d.f. = 2, 116, $p < 0.0001$; age: $F = 22.94$, d.f. = 2, 116, $p < 0.001$; trial: $F = 3.26$, d.f. = 2, 116, $p = 0.042$). Interactions between diet and trial were significant (diet × trial: $F = 3.64$, d.f. = 4, 116 $p = 0.008$) but between diet and age and age and trial were not ($F = 0.95$, d.f. = 4, 116, $p = 0.95$; $F = 0.18$, d.f. = 4, 116, $p = 0.11$). Because the effects of trial were significant, separate repeated measures ANOVA were conducted to compare acini area and age among the diets.

Pairwise comparisons of means using Bonferroni adjusted confidence intervals (*post hoc* comparisons of the repeated measures analysis of variance) indicated that in trial-1, acini were larger in workers fed pollen than in the controls ($p = 0.034$) (Fig. 3). The acini from bees fed protein supplement did not differ from pollen or controls ($p > 0.05$). In trial-2, acini of workers fed pollen did not differ from those fed protein supplement or controls

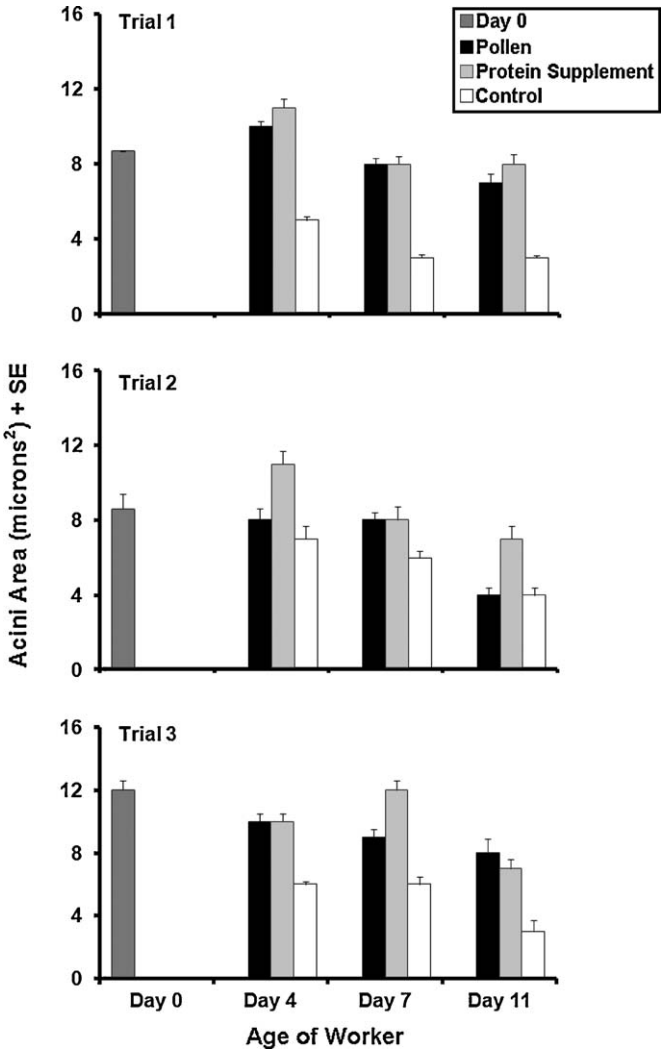

Fig. 3. Average area of 5 hypopharyngeal gland acini that were measured in 15 worker honey bees that were 4, 7, or 11 days old. The bees were kept in cages and fed pollen, protein supplement or water and high fructose corn syrup alone (control). In trial-1, acini were larger in workers fed pollen than in the controls ($p = 0.034$). Acini from bees fed protein supplement did not differ from pollen or controls ($p > 0.05$). In trial-2, acini size did not differ between bees fed pollen and those fed protein supplement or controls ($p > 0.05$). However, bees fed protein supplement had significantly larger acini than controls ($p = 0.035$). In trial-3, the acini were larger in workers fed pollen or protein supplement compared with controls ($p = 0.001$, 0.001 respectively). In all trials, acini in day-4 workers were significantly larger than those in day-7 or day-11 workers (Trial-1: $p = 0.01$, $p = 0.006$ respectively; Trial 2: $p = 0.003$, $p = 0.0001$; Trial 3: $p = 0.005$, $p = 0.038$).

($p > 0.05$). However, bees fed protein supplement had significantly larger acini than controls ($p = 0.035$). In trial-3, the acini were larger in workers fed pollen or protein supplement compared with controls ($p = 0.001$, 0.001 respectively). In all trials, acini in day-4 workers were significantly larger than those in day-7 or day-11 workers (Trial-1: $p = 0.01$, $p = 0.006$ respectively; Trial 2: $p = 0.003$, $p = 0.0001$; Trial 3: $p = 0.005$, $p = 0.038$).

3.4. Interrelationships of consumption, protein concentration and acini size

Regression analyses were conducted to test for a relationship between diet consumption per cage and protein concentrations detected in bees. There was not a significant correlation at $p > 0.05$ between the amounts of either pollen or protein supplement consumed and the protein concentrations we detected in the bees.

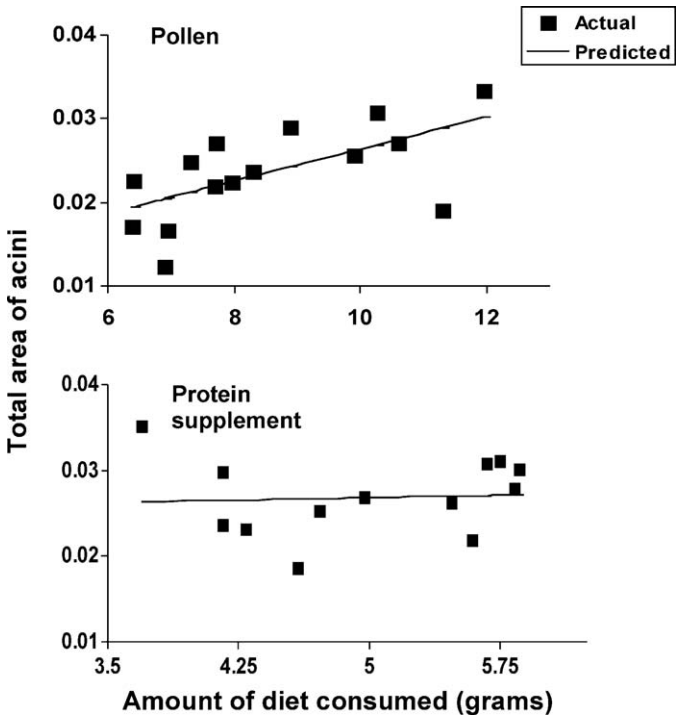

Fig. 4. The relationship between the amount of pollen or protein supplement consumed during an 11-day period and the sum of measurements of hypopharyngeal gland acini taken on days 4, 7 and 11 after emergence. Measurements were made using 5 acini from 15 workers at each time interval. The regression equation for pollen consumption vs. acini size is: $y = 0.00704 + (0.00192x)$; $r^2 = 37.9$, p (slope) = 0.015, p (y-intercept) = 0.26. The regression equation for protein supplement consumption vs. acini size is: $y = 0.0249 + (0.00036x)$; $r^2 = 0.4$, p (slope) = 0.835, p (y-intercept) = 0.013.

We also tested for a relationship between the total amount of diet consumed by bees per cage, and the total area of acini in workers. We found a significant correlation between the amount of pollen consumed and the size of acini (Pearson correlation = 0.62, $p = 0.015$). Acini area increased with greater pollen consumption, and 38% of the variation in the size of acini was due to the amount of pollen they consumed (Fig. 4). The amount of protein supplement consumed however, was not correlated with acini size (Pearson correlation = 0.061, $p = 0.835$).

3.5. Validation of the assay for DWV quantification

Among seven viruses screened for infections by conventional RT-PCR method, DWV was the only virus found. Therefore DWV was selected for further quantification of virus concentrations in bees fed with different diets, using SYBR Green qRT-PCR method. The standard curves demonstrated linear progression of the amplification and a linear relationship was observed between the amount of input total RNA and the C_t values for both DWV ($y = -2.558 \times \log(x) + 17.76$, $R^2 = 0.977$, Eff. = 155.2) and β-actin ($y = -2.628 \times \mathrm{Log}(x) + 21.59$, $R^2 = 0.99$, Eff. = 140.2%) within the concentration range tested. The closing slope values of two standard curves indicate approximately equal amplification efficiency for both target and endogenous control. Therefore, the comparative C_t method for the relative quantification of DWV in the study was valid.

SYBR Green dye intercalates with any double-stranded DNAs and emits fluorescence signals, thus both specific and non-specific dsDNA PCR products are detected. The presence of a single homogeneous melt peak at 78.5 °C for all sample reactions indicates a single PCR product was amplified in these samples and confirms specific amplification of DWV, demonstrating that

the real-time qPCR assay was of high specificity and the data from this reaction are meaningful for virus quantification. While the β-actin amplification plot displayed similar C_t values reflecting that the same amount of total RNA was used for each sample tested, there was a significant difference in Ct values among bees fed with different diets at different time points post treatment, indicating variable virus levels in bees of different treatment groups

3.6. Relative quantitation of DWV in bees

The relative concentrations of DWV in bees that received different diet treatments were estimated from the difference between the C_t value (ΔC_t) of DWV and β-actin. The lower the ΔC_t value, the higher the concentration of virus that was detected in the sample. Symptoms of DWV infection (i.e., malformed wings and decreased body size) were not present in any of the examined workers; therefore the virus titers were not sufficient for manifestation of disease symptoms.

The group of day-11 bees that were fed pollen had the highest ΔC_t value representing the lowest level of DWV infection and thus was chosen as a calibrator. The concentrations of all other samples were estimated relative to that of the calibrator. The ΔC_t value of individual samples relative to the calibrator was described as $2^{-\Delta\Delta C_t} = 2^{-(\Delta C_t \text{ target} - \Delta C_t \text{ calibrator})}$.

The proportion of bees where virus was detected did not differ among diet treatments until after 7 days of feeding. At this time, the proportion of bees with virus was greater in controls compared with those fed pollen or protein supplement (pollen: $z = -3.24$, $p = 0.001$; protein supplement: $z = -2.31$, $p = 0.02$). On day 11, virus was detected in fewer bees that were fed pollen compared with controls ($z = 3.24$, $p = 0.001$); however, control bees and those fed protein supplement did not differ ($z = -1.63$, $p = 0.10$). The proportion of bees where virus was detected did not differ between those fed pollen or protein supplement at any sampling interval ($p > 0.05$).

Changes in DWV concentrations from days 0 to 11 were affected by the diet fed to the bees (Fig. 5). The concentration of DWV in 4-day old bees increased in controls and those fed protein supplement, but decreased in bees fed pollen. By day 11, the lowest concentrations of DWV were detected in bees fed pollen or protein supplement, and the highest concentrations in controls.

4. Discussion

Protein supplements are fed to honey bee colonies when pollen is unavailable. We found that bees fed a protein supplement had protein levels and HPG development comparable to those fed pollen. Less of the protein supplement was consumed than the pollen, yet similar protein levels and acini development occurred in bees fed either diet. The nutrition provided by the pollen and protein supplement also appeared to influence DWV titers. Concentrations of DWV decreased dramatically in bees fed pollen or protein supplement compared with controls. Because this study was done using caged bees, we could not measure the effects that brood and nestmate interactions at a colony level might have on the factors we examined. However, the results can provide insights into the potential of protein supplements to alleviate the effects of protein deprivation on workers and how this might translate into colony growth.

Fig. 5. Fold difference of deformed wing virus (DWV) in bees pre- and post-diet treatments. The virus concentrations in bees were expressed as fold difference compared to the level of virus of a calibrator. The group of 11-day old bees fed pollen was chosen as a calibrator (indicated by a star) because they had the lowest ΔC_t value, and thus represented the lowest level of DWV infection. The results of fold difference were calculated using the formula $2^{-\Delta\Delta C_t} = 2^{-(\Delta C_t \text{ target} - \Delta C_t \text{ calibrator})}$. The star represents undetectable virus levels.

Protein levels and acini size were similar in bees fed either pollen or protein supplement though consumption rates differed between them. A possible explanation might be that the rate of digestion for either diet might have been limited such that consuming more did not cause measurable increases in protein levels. This might be particularly true for pollen which is difficult to digest due to the waxy wall surrounding pollen grains (Piffanelli et al., 1998; Klungness and Peng, 1984; Peng et al., 1985; Crailsheim and Stolberg, 1989). In colonies, bees store pollen in cells where it is fermented and pre-digested by the action of microbes (Gilliam, 1979a,b; Gilliam et al., 1989). In our study, the protein that bees were able to obtain from the pollen was solely from digestion in the gut. This might limit the effectiveness of protein supplements containing pollen in colonies. Though they might be consumed at higher rates, after a point the increased consumption might not translate into greater nutritional benefits.

All workers had developed HPG within the first 24 h after emergence. Workers were not exposed to brood, thus it appears that enlargement of acini at least until day 4 is dependent upon the age of the worker rather than the presence of brood. Similar results were reported in a study where acini were measured in worker bees housed in colonies without open brood (Crailsheim and Stolberg, 1989). However, the lack of brood exposure might have affected additional growth of acini as the bees aged. In colonies, the activity of HPG declines when brood is removed because larvae provide workers with chemical signals that induce protein synthesis in HPG (Brouwers, 1983; Huang et al., 1989). However, the glands also have a food storage function. In winter, acini can be well developed though their protein synthesizing activity is low (Moritz and Crailsheim, 1987; Hrassnigg and Crailsheim, 1998). The bees in our study might resemble winter bees in colonies when little or no brood is present yet HPG are developed. Our observations suggest that protein supplement fed in the fall might be stored in bees and provide a ready supply of protein when brood rearing resumes in winter and pollen is not available or the colony is confined due to weather conditions.

DWV levels in bees that did not receive supplemental protein increased as the bees aged and were significantly higher than in those fed either pollen or protein supplement. While the specific role of protein in the activation of the immune system has been characterized and documented in humans and other animals (Kaminogawa and Nanno, 2004; Calder and Yaqoob, 1999; Houdjik et al., 1998; Zheng et al., 2006; Gianotti et al., 1999), relatively little is known concerning a role of protein in the immune response of honey bees. Others have reported though, that alleviating protein stress in colonies by supplemental protein feeding can mitigate the effects of parasitism by Varroa mites (Janmaat and Winston, 2000) and reduce the toxicity of some pesticides to honey bees (Wahl and Ulm, 1983). Our results suggest that supplemental protein feeding might slow the replication and spread of DWV. However, the effects of diet

on bees in cages especially as it relates to reduction in the impact of disease are not always repeated in similar studies in colonies where other stress factors can contribute to the overall health of workers (Mattila and Otis, 2006b). Our study needs to be repeated in colonies before the connection between supplemental protein and changes in virus titers can be fully confirmed.

Honey bee populations have been declining over the past 40 years (NRC, 2007), and recently there have been huge losses of managed colonies from CCD (vanEngelsdorp et al., 2007, 2008). Perhaps at the heart of many colony deaths is poor nutrition that acerbates the stress bees experience from parasitic mites, disease and environmental toxins. Management tools to reduce mite populations or reduce disease are limited and not always reliable or effective. However, one factor that can be managed effectively is colony nutrition. Our study indicates that protein supplements can closely resemble pollen in nutritional value and because of their effects on protein concentrations and HPG development can play a significant part in reducing colony losses.

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